Mass Spectrometry for Rapid Characterization of Microorganisms

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MALDI, ESI, tandem MS, bacteria, viruses, spores, proteomics, bioinformatics, proteolysis

Abstract

Advances in instrumentation, proteomics, and bioinformatics have contributed to the successful applications of mass spectrometry (MS) for detection, identification, and classification of microorganisms. These MS applications are based on the detection of organism-specific biomarker molecules, which allow differentiation between organisms to be made. Intact proteins, their proteolytic peptides, and nonribosomal peptides have been successfully utilized as biomarkers. Sequence-specific fragments for biomarkers are generated by tandem MS of intact proteins or proteolytic peptides, obtained after, for instance, microwave-assisted acid hydrolysis. In combination with proteome database searching, individual biomarker proteins are unambiguously identified from their tandem mass spectra, and from there the source microorganism is also identified. Such top-down or bottom-up proteomics approaches permit rapid, sensitive, and confident characterization of individual microorganisms in mixtures and are reviewed here. Examples of MS-based functional assays for detection of targeted microorganisms, e.g., *Bacillus anthracis*, in environmental or clinically relevant backgrounds are also reviewed.

1. INTRODUCTION

Tandem MS: precursor ions are selected by a mass spectrometer and allowed/induced to fragment, then analyzed by a second mass spectrometer

The worldwide threat to human health from existing as well as emerging infectious pathogens has not diminished despite spectacular advances in medicine. Thus, the need for novel molecular-level technologies for rapid and confident microorganism detection, identification, and characterization is constantly growing. Current technologies based on antibody recognition or DNA detection after polymerase chain reaction (PCR) amplification typically require hours to yield results (1). However, mass spectrometry (MS) possesses several unique features that make it an attractive complementary technology for microorganism detection, identification, and characterization.

First and foremost, MS is broad band: Its capability to detect organisms is not restricted to prespecified targets, whereas most other molecular detection technologies must rely on molecular recognition events by either antibodies or DNA primers and probes to selectively bind predetermined and specified targets. Second, MS is rapid: A typical experiment from sample collection and preparation to final result requires as little as a few minutes. In contrast, days are needed for (often retrospective) detection and verification via the classical microbiology methods that rely on microorganism culture. Third, MS is sensitive: A signal with a sufficient signal-to-noise ratio can be generated from a sample containing less than 10^4 microorganisms. A variety of sample-collection and sample-processing modules for sampling of microorganisms and their constituents from different environments—from aerosols to biofluids—can be interfaced to MS instruments. Finally, tandem MS in conjunction with chromatography and bioinformatics is the most rapidly growing analytical technology in the postgenomic era, fueling rapid global advances in proteomics. Most proteomics tools, developed initially for protein characterization, can be successfully adapted for rapid characterization of microorganisms.

For more than 30 years, the capabilities of MS have been utilized to characterize bacteria, bacterial spores, and other kinds of microorganisms with minimal sample preparation and without fractionation, centrifugation, or chromatography (2–9). The development of rapid MS methods for microorganism detection has been driven, in large part, by government interest in the identification of biowarfare agents in the "detect to protect" time frame of under several minutes and the "detect to treat" time frame of several hours (10, 11). The capability of MS to rapidly characterize microorganisms has potential applications in a number of areas beside medical diagnostics, biodefense, and homeland security, such as environmental monitoring, agricultural stewardship, food quality control, occupational safety, and culture typing.

Every molecule has a mass, an immutable intrinsic property reflecting its elemental composition. The masses of intact molecular ions and their fragments can be facilely determined by various kinds of MS. In addition, in tandem MS the intact molecular ions can be internally excited to produce fragment ions, which provide information about molecular structures and sequences. The current paradigm for rapid MS identifications of intact microorganisms relies on determination of the masses of unique biomarker molecules from experimental mass spectra of intact organisms or their extracts. This paradigm can be traced back to Anhalt and Fenselau (2) who

demonstrated (*a*) that biomolecules from different pathogenic bacteria, introduced intact in a mass spectrometer, could be vaporized and directly ionized; (*b*) that these molecules could be structurally identified; and (*c*) that taxonomic distinctions can be made based on the characteristic mass spectral "fingerprint" signatures for individual organisms. The experimentally observed signatures can be related to either intact biomarker molecules or their fragments, and each organism-specific signature can be derived experimentally by acquiring mass spectra for a particular microorganism under a variety of conditions. Alternatively, these signatures can be deduced/derived by means of bioinformatics. **Figure 1** illustrates the confluence of experimental and theoretical analyses in these strategies.

In the following sections, we discuss biomarkers that are predominantly peptides or proteins. Peptides and proteins are more abundant in microbial cells compared to other classes of molecules. In addition, proteins are ionized efficiently and resolve some of the ambiguities of DNA sequences (6, 12). At the same time, protein sequences are linked to gene sequences and are therefore more characteristic biomarkers than lipids and metabolites. We point out that there are MS-based applications in microbiology utilizing biomarkers other than proteins, such as DNA (13) and 16s

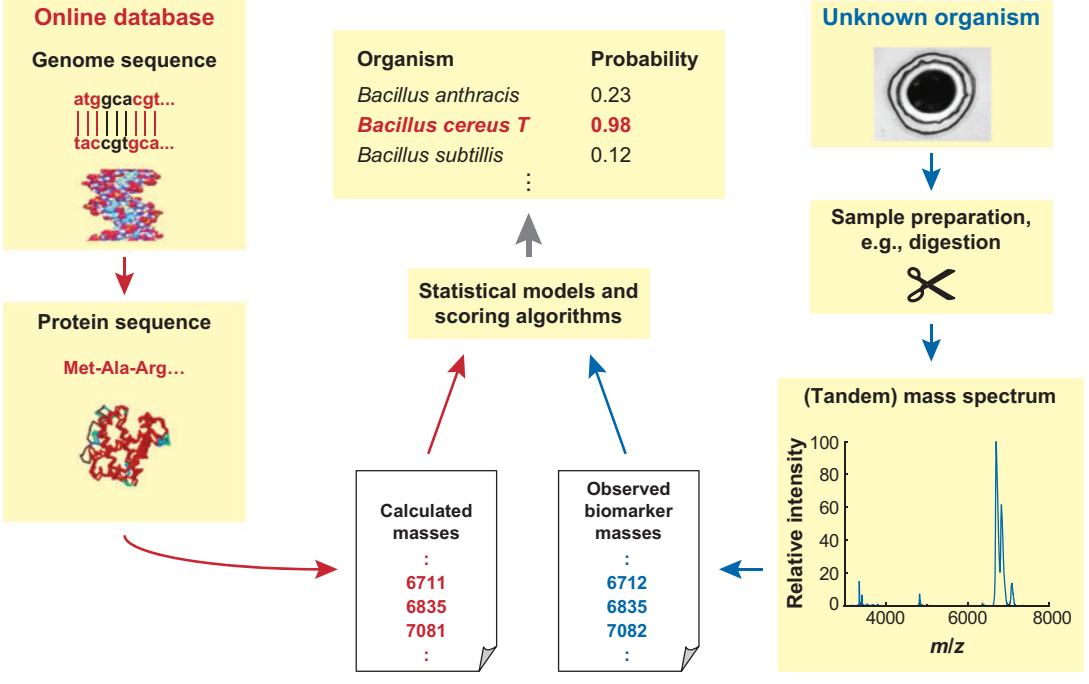

Figure 1

Strategy for rapid characterization of microorganisms by mass spectrometry (MS) and bioinformatics. An experimental mass spectrum of an unknown is matched against the in silico–predicted masses of organisms with sequenced genomes. The "best" match is determined by a statistical algorithm.

RNA (14, 15). Low-mass molecules and molecular fragments have also been reported as MS-based biomarkers for microorganism detection and identification—e.g., heme as a biomarker for malaria (16)—or in bacterial spore forensics (17), phospholipids (18) or fatty acid methyl esters (19).

TOF: time of flight

Electrospray ionization (ESI): multiply charged biomolecular ions are generated by transporting solution containing analyte through a capillary needle biased at high voltage

Matrix-assisted laser desorption/ionization (MALDI): ionization method for large nonvolatile biomolecules, wherein the analyte is mixed with a matrix, then dried and irradiated with laser pulses

AP: atmospheric pressure

1.1. Instrumentation

A number of custom-built and commercially available ion analyzers have been demonstrated for rapid analysis of proteins from unfractionated bacteria, notably time of flight (TOF), Fourier transform, and ion trap analyzers. Small TOF- and ion trap–based instruments have been designed for field portability (e.g., 19–24), whereas larger instruments with higher mass-resolving power have been proposed for work in reference laboratories (25–29). Providers of fieldable systems have incorporated small and rugged pumps and detectors, as well as devices for sample collection and processing. The major advances that have enabled reliable rapid analysis of microorganisms are two techniques particularly suited for ionization and transfer into the gas phase of large nonvolatile biomolecules such as intact proteins: laser desorption and electrospray (30). Successful characterization of an unfractionated nonenveloped virus using electrospray ionization (ESI) was reported in 1996 (31).

Challenges to broadening the application of ESI in this area have included mechanical clogging and obstruction of charge state deconvolution by the presence of multiple proteins, each with a distribution of charge states. Matrix-assisted laser desorption/ionization (MALDI) generates mostly singly charged ions, and thus MALDI spectra accommodate larger numbers of proteins than those of ESI. Both MALDI and ESI are selective: Depending on their intrinsic properties, some proteins in a mixture are more easily ionized than others. MALDI mass spectra from intact microorganisms are not readily reproducible, being sensitive to sample:matrix ratios, the presence of contaminants, and the irregular surfaces of bacterial samples (32).

Newer techniques for direct sample ionization from ambient sources have also been interfaced with different types of mass spectrometers for analysis of intact bacteria or rapidly digested bacterial extracts. These ionization techniques are capable of interrogating a sample directly in the atmosphere before transferring the ions into the instrument for mass analysis. They include atmospheric pressure (AP) MALDI (23, 33), desorption electrospray ionization (DESI) (34), and direct analysis in real time (DART) (35).

1.2. Sample Preparation

Characterization of environmental or clinical microorganisms usually requires enriching or purifying the bacteria and viruses obtained from air, water, foodstuffs, urine, blood, et cetera. The use of antibodies offers a universal method for separating and enriching targeted species (36, 37). The use of lectin/carbohydrate affinity binding has also been demonstrated (38, 39); this technique offers a broad-range, less selective recovery. Microorganisms have been recovered from air (the cleanest medium) on filters, in aqueous solutions, and directly onto MALDI sample holders via

particle impactors. In the latter case, the sample is cleaned up (for instance, separated from fine sand) by transmitting particles only in a preselected size range. Several laboratories have undertaken analysis of single particles such as bacterial rafts introduced directly into mass spectrometers (40–43). Coating particles in flight is proposed to permit MALDI analysis of protein biomarkers (40, 41, 43).

Intact bacteria have been separated by capillary electrophoresis (44) and field flow fractionation (45), which are compatible with subsequent mass spectrometric analysis, albeit in a longer time frame compared to direct sample deposition. A small virus (MS 2) as well as individual *Escherichia coli* cells have been weighed intact (and nonroutinely) by MS (46, 47). Exposure to MALDI matrix solutions lyses many bacteria types in situ (i.e., on the sample holder) and the released proteins cocrystallize with the matrix. MALDI matrix solutions and most electrospray solutions contain both aqueous acid and organic solvents. Rapid extraction of protein biomarkers from bacterial spores and some viruses may be achieved by the use of strong acid or organic solvents (12, 48–54). An automated system would be expected to provide adequate conditions for effective cell lysis and also for biomarker ionization.

Below we describe several ways in which proteins released from or accessed in intact cells are hydrolyzed in situ to peptides for proteomic analysis. Immobilized trypsin can be used in high concentrations for rapid proteolysis (53, 55, 56), and microwave-assisted residue-specific acid cleavage has recently been demonstrated (57–59).

2. RAPID ANALYSES BASED ON INTACT PROTEINS FROM UNFRACTIONATED BACTERIA

2.1. Exploiting Molecular Masses of Intact Biomarkers

Most MALDI spectra of proteins from intact microorganisms contain intense peaks below *m/z* 25,000. These spectra are sufficiently distinctive to establish species-characteristic fingerprint libraries (60). Both the mass range and species differences are illustrated in **Figure 2**. To identify the microorganism, experimental MALDI mass spectra from unknowns are compared with such reference libraries. However, MALDI MS of intact microorganisms is influenced by many experimental factors, such as individual protein biomarker solubility, variation of expression levels due to culture time and media, ionization efficiencies for different biomarker molecules (e.g., as a function of matrix:biomarker protein ratios), variation in laser pulse energy, and instrument-dependent detection efficiency (32, 61, 62). A round robin study of MALDI mass spectral reproducibility from intact microorganisms has been performed in three independent laboratories with three different commercial instruments using identical aliquots of *E. coli* culture, matrix, and calibration standards as well as automated data processing and analysis algorithms (63). Only 25% of the biomarker ions attributed to this bacterium were found in common by all three laboratories, whereas more than 50% were observed in spectra from only one of the three laboratories.

Several fingerprint libraries are available commercially, as are directions on how to culture and prepare samples to ensure reproducible, searchable spectra. Numerous

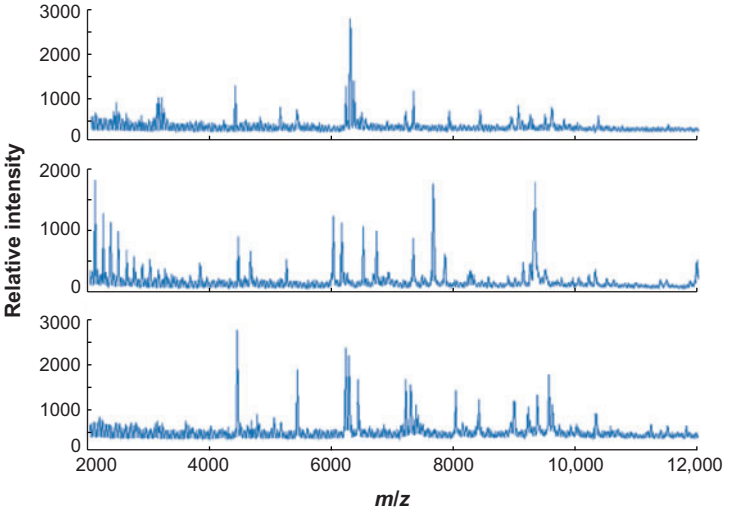

Figure 2

Matrix-assisted laser desorption/ionization time-of-flight mass spectra from three intact bacterial species: *Escherichia coli* (*top*), *Pseudomonas stutzeri* (*center*), and *Pseudomonas aeruginosa* (*bottom*). The range of positive ions from *m/z* 2000 to 12,000 is plotted. Adapted with permission from Reference 60. Copyright 2002, American Chemical Society.

reports advocate the use of this approach for environmental monitoring, microbial forensics, and potential clinical applications (12, 64–72). A standardized method, which includes culture time and medium, number of passages, and matrix type and concentration, has been proposed for MALDI MS–based strain typing of clinical isolates of methicillin-resistant *Staphylococcus aureus* (66). MALDI MS for cultured environmental samples has been compared with PCR for rapid discrimination between closely related strains of *E. coli* and for tracking bacterial contamination in surface water (71). Although MALDI MS reproducibility appears to be lower than that of PCR, the MS approach was superior in correctly assigning *E. coli* isolates to a specific contamination source.

Difficulties in reproducing the MALDI mass spectra of intact microorganisms, without the possibility of culturing or controlling other experimental parameters, have prompted the development of a bioinformatics approach based on proteome database queries (4, 73–78). In this approach, peaks in the spectra need not be reproducible if the protein masses can be related to the relevant genome (73). The experimentally observed biomarker masses are matched to predicted suites of protein molecular masses from a proteome database. A bioinformatics-based tool has been developed to introduce additional constraints on candidate protein biomarkers by determining the number of methionine residues in a sequence from the mass shifts before and after rapid in situ sample oxidation (79). The method has been predominantly applied to microorganism identification at the genus and species levels. Depending on database availability, even strain specificity has been reported for *Campylobacter* (80) and *Bacillus* (81).

The bioinformatics method has been strengthened with the introduction of hypothesis testing to quantify the significance of microorganism identifications (74). For that purpose, the probability (p-value) for a random match between an experimentally observed biomarker mass and the calculated mass of an unrelated database protein is introduced. The lower probability reflects a lower likelihood for misidentification due

to accidental matches. Therefore, lower p-values provide more confident overall microorganism identifications. Both theoretical analysis of the bioinformatics approach and statistical modeling (in silico MS) demonstrate the dependence of p-values on experimental parameters, e.g., mass accuracy, number of experimentally observed independent biomarkers, and database properties (74). Increasing the mass accuracy of the experimentally observed biomarkers by external/internal calibration (82), higher resolution mass measurements (27), and/or isotope depletion improves confidence in microorganism identification.

We identify several important features and specific requirements for a proteome database to be used successfully in microorganism identification by mass spectrometry. The first important feature of the database is its completeness, characterized by the number of organisms with sequenced genomes. Obviously, for an organism to be identified by this approach its genome has to be sequenced (at least partially). Currently there are more than 550 bacteria and archaea and more than 1800 reference virus genome sequences publicly available (83).

Another important feature of the database is its fidelity, namely the correlation (or lack thereof) between observed and predicted biomarker masses from the amino acid sequences. The observed and predicted masses of intact proteins are different if posttranslational modifications (PTMs) are present. The occurrence of the most common PTM in prokaryotes—loss of the N-terminal Met (start codon)—can be predicted with a large degree of certainty from the penultimate N-terminal amino acid (75). Microorganism identification (evaluated from the p-values) is improved by at least an order of magnitude if this PTM is accounted for (75).

The third important database feature is biomarker density: the number of database proteins per unit mass interval for a given microorganism. Both statistical modeling (74, 77) and experimental results (76) predict that reducing the database density by rationally constraining the entire proteome of a sequenced organism allows its successful identification from its experimental mass spectrum at the mass accuracy typical for linear TOF instruments. For instance, including only ribosomal proteins (a class of highly expressed proteins) in a microbial biomarker database reduces the number of biomarkers in the range of 4 to 20 kDa by more than two orders of magnitude. In a blind study, microorganisms represented in this database were correctly identified from their experimental MALDI spectra 100% of the time at the 95% confidence level, with no incorrect identifications (76). In addition, a truncated protein biomarker database has been created using only bioinformatics tools and microbial genome sequence information (77). That approach relies on the correlation between the statistics of synonymous codon usage in a gene and the expression level of the protein coded by that gene (84, 85). Truncated databases have also been constructed using mass spectrometry to identify highly expressed biomarkers in an organism (86, 87). Data compression from a two-dimensional array of high-performance liquid chromatography (HPLC) retention times and electrospray mass spectra into a one-dimensional mass/intensity spectral format allows the creation of biomarker profiles for bacteria from electrosprayed extracts of intact proteins (87). Microorganism-specific search algorithms based on experimental mass spectra, proteomics, and bioinformatics are available on a publicly accessible website (88).

PTM: posttranslational modification

HPLC: high-performance liquid chromatography

2.2. Protein Fragmentation by Tandem Mass Spectrometry

SASP: small acid-soluble spore protein

A complement to the analysis of proteins ionized from unfractionated bacteria is offered by instruments that can fragment intact proteins to provide sequence-specific fragment ions. In this "top-down" proteomics approach (89), an intact protein is identified by deducing its partial amino acid sequence after fragmentation in a tandem MS experiment and subsequent homology search. MALDI and top-down proteomics have recently been demonstrated on a tandem TOF instrument for the rapid and high-confidence identification of intact *Bacillus* spore species (29). Unlike ESI/FT-ICR (Fourier transform ion cyclotron resonance), MALDI TOF top-down proteomics does not require protein biomarker enrichment and separation prior to analysis. It can directly interrogate intact microorganisms, both pure and in mixtures, and switching from MS to tandem MS mode takes just a few seconds. Dissociation of precursor protein ions results in sequence-specific backbone cleavages, with spectra dominated by ions formed by cleavages on the C-terminal side of aspartic or glutamic acid residues.

To identify the precursor ion, the spectra are compared with tandem spectra generated in silico from all the protein sequences in a proteome database, whose masses correspond to the intact precursor ion mass observed. By inference, the source microorganism is then identified, based on the identification of one or more individual protein biomarker(s). For example, the major biomarker ions between *m/z* 6000 and 9000 observed in MALDI spectra of mixtures of intact *Bacillus* spores can all be unambiguously identified (29). From here, the presence of, for instance, intact *B. anthracis* spores in the mixture can be confirmed. **Figure 3** illustrates the fragmentation of the protein with MH+ at *m/z* 6680 in the MALDI spectrum of a mixture of *B. anthracis* Sterne spores and *B. cereus* T spores. Following the procedure outlined in Reference 29, a small acid-soluble spore protein (SASP) from *B. anthracis* is unambiguously identified from the tandem spectrum (p-value 7.4×10^{-10}), thus indicating the presence of intact *B. anthracis* spores in the sample. The MALDI tandem MS spectra of intact biomarkers are fairly reproducible, and library fingerprint matching of such tandem mass spectra can be implemented for intact microorganism identification. This may be particularly advantageous when the sequence of the microorganism genome is not available, or when only a few protein biomarkers, such as virus coat proteins, are present in a regular mass spectrum.

Top-down proteomics, using ESI on an ion trap instrument to study intact SASPs extracted from *Bacillus* spores, has recently been applied to grouping ten different *B. cereus* strains into two clusters, one closely and one distantly related to *B. anthracis* (90). The strains from the closely related cluster contained a major SASP with only a single amino acid substitution (close to either the C-terminus or the N-terminus). The more distantly related cluster displayed both amino acid substitutions, as previously observed by MALDI TOF MS (51, 77). Interestingly, a *B. cereus* isolate from a patient with severe pneumonia (an anthrax-like disease) has been grouped into the more distantly related cluster based on masses of observed SASPs alone.

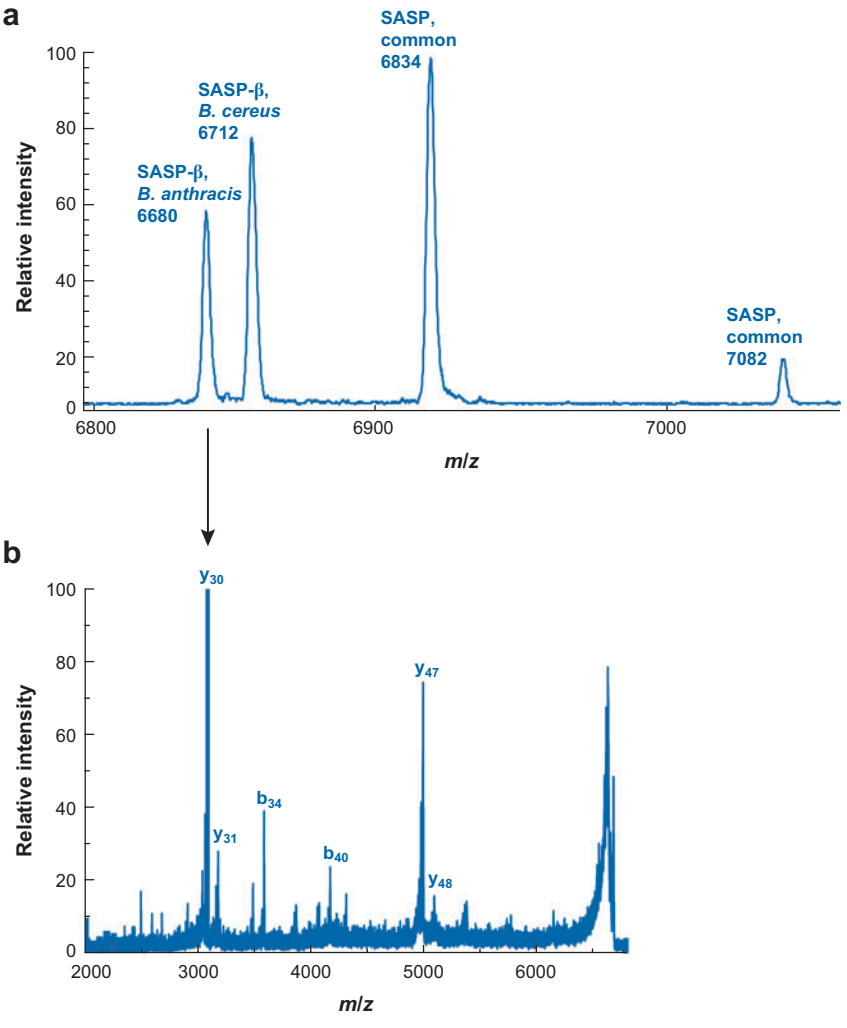

Figure 3

(*a*) Matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) mass spectrum of a 1:1 mixture of intact *Bacillus cereus* T spores and *Bacillus anthracis* Sterne spores. (*b*) Tandem mass spectrum (MALDI TOF/TOF) of the precursor ion at *m/z* 6680. SASP, small acid-soluble spore protein.

3. RAPID CHARACTERIZATION OF BACTERIA BASED ON PEPTIDES FROM BIOMARKER PROTEINS

3.1. Sequence Tags

The identification of one or more proteins from partial sequences readily allows the identification of a bacterium or closely related family at the species level. Parallel to the top-down approach, a bottom-up strategy (91) has also been adopted from proteomics for the rapid identification of bacteria and spores. Proteins fractionated from lysed cells, or accessed in situ in automated rapid strategies, are cleaved to

peptides, which are then fragmented by collisionally induced dissociation (CID) or laser-induced dissociation. These tandem mass spectra provide the basis for identifying the peptides. Identified peptides provide identification of proteins and, in turn, proteins provide identification of microorganisms. This strategy has been implemented using both MALDI (92–94) and electrospray ionization (6, 28, 95–99). **Figure 4*a*** presents the MALDI TOF spectrum of a 1:1 mixture of *Bacillus anthracis* Ames spores and *Bacillus subtilis* 168 spores, which had been treated for 90 s with hot formic acid. Both molecular ions and peptide ions were detected. **Figure 4*b*** presents a portion of the spectrum with an expanded abscissa. In **Figure 4*c*** we see the tandem mass spectrum obtained from the MH+ peak at *m/z* 2462 using laser-induced dissociation (58). From this spectrum, the peptide was identified as originating from extracellular antigen 1 surface-layer protein in *B. anthracis*. All spore components of the mixture were identified by considering the molecular masses and tandem spectra of the other peptides and small proteins observed in **Figure 4*a*,*b***.

Collisionally induced dissociation (CID): ions in a mass spectrometer are dissociated to fragments/neutrals as a result of nonthermal collisions with inert gas neutral atoms/molecules

One benefit of interrogating peptides is the increased mass accuracy and sensitivity of ion analyzers and detectors for smaller ions such as peptides compared to protein molecular ions. This strategy provides access to heavier proteins (via their peptides) and has been suggested as one solution to the challenges of identifying components of mixtures of microorganisms (53, 92, 93, 96) and of recognizing plasmid insertion in genetically engineered bacteria (94). When this bottom-up approach is implemented in a rapid automatable MALDI strategy, residue-specific cleavage of biomarker proteins is required as an extra step and is carried out enzymatically or chemically in situ. The strategy works best when only a limited set of proteins is preferentially solubilized.

Traditional bottom-up proteomics approaches, which involve extensive sample preparation and peptide analysis by chromatography/ESI/tandem MS, have also been successfully applied to the characterization of individual microorganisms in often complex mixtures (6, 95–99). Although these methods are slower than the rapid MALDI approach, one- or two-dimensional chromatography improves considerably the MS capability to identify many individual peptides. Shotgun proteomics and dedicated software have been recently applied to *B. anthracis* strain differentiation (99). Selective interrogation of subsets of targeted characteristic peptides has been proposed in both MALDI (94) and electrospray (96) workflows in order to reduce the overall analysis time.

3.2. Small Acid-Soluble Spore Proteins

The SASP family of proteins constitutes up to 15% of the mass of *Bacillus* spores. These basic proteins can be selectively solubilized in acid, and they are readily protonated to provide strong signals in MALDI and electrospray spectra. They have been studied by many researchers interested in protein-based identification of *B. anthracis* spores, and numerous publications have considered the question of how specific the biomarkers provided by the SASP family are (e.g., 11, 12, 29, 77, 92, 94, 100). A survey of

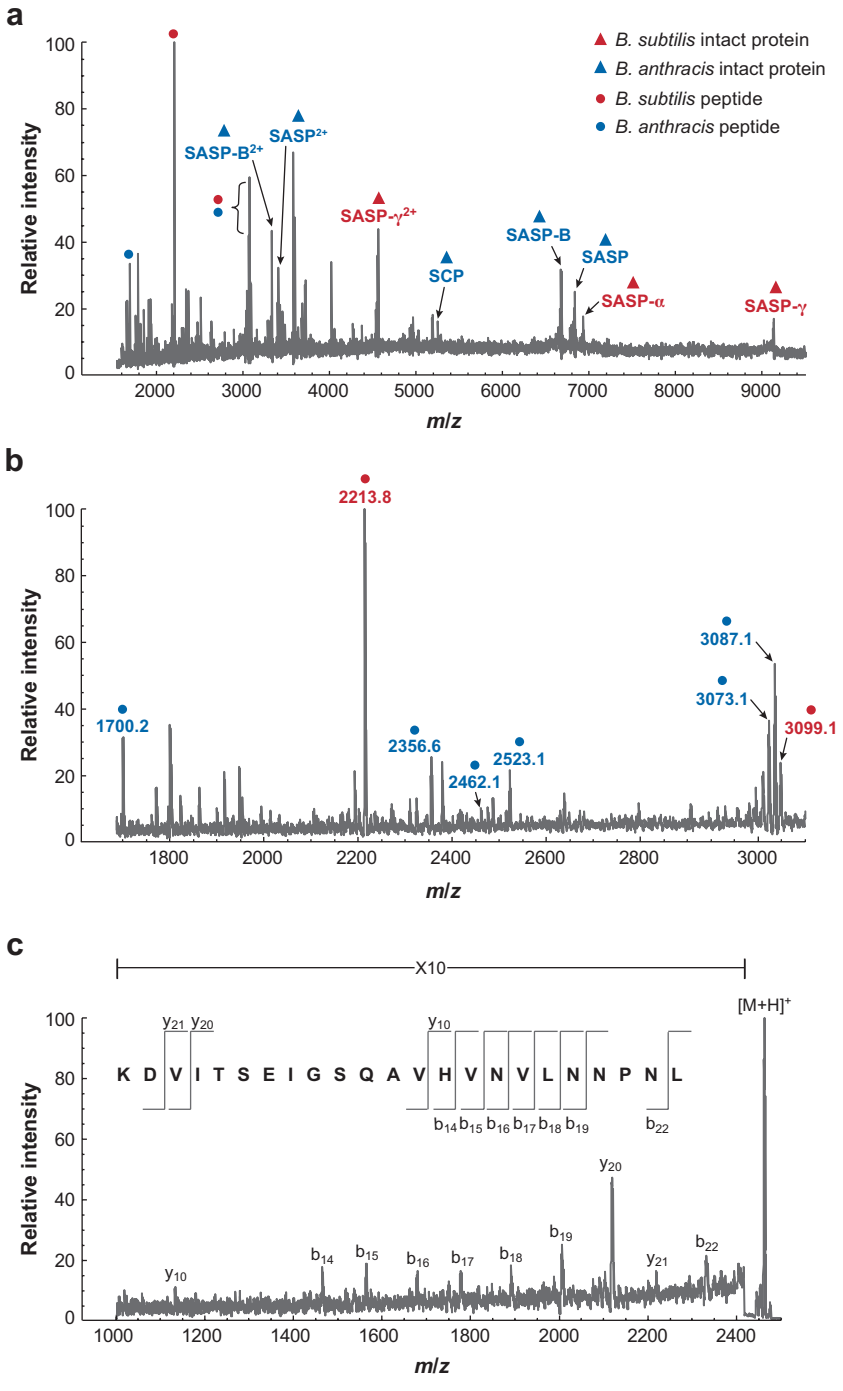

Figure 4

(*a*) Matrix-assisted laser desorption/ionization mass spectrum of proteins and peptides obtained by 90-s microwave-assisted acid digestion of a 1:1 mixture of *Bacillus anthracis* Sterne spores and *Bacillus subtilis* 168 spores. (*b*) Spectrum from panel *a* expanded between *m/z* 1700 and 3100. (*c*) Tandem mass spectrum obtained by laser-induced dissociation of a *B. anthracis* Sterne peptide with MH+ at *m/z* 2462. SASP, small acid-soluble spore protein. Reproduced with permission from Reference 58. Copyright 2006, American Chemical Society.

publicly available genomes of ten *B. anthracis* strains has evaluated the specificity of the SASPs and their peptides obtained by either immobilized trypsin or aspartate-specific chemical digestion to characterize each strain (94). Five of the eight *B. anthracis* SASPs have exactly the same sequences as SASPs from different, albeit closely related species, such as *B. thuringiensis*, *B. cereus*, and others. Two of the remaining SASPs are distinct in mass from the respective SASPs in the other two species. However, each individual tryptic peptide from these SASPs can be found in at least one other *B. cereus* or *B. thuringiensis* strain (94). The sequence of only one SASP (γ) indicates that both the molecular ions and peptide products allow the source of this protein to be distinguished. As has been shown (18, 58, 66, 100), use of the SASP family for species identification is optimized when measurements can be made of both the molecular masses and critical peptides or microsequences.

In general, the probability for positive matching of a protein but false matching of a microorganism is lower in top-down proteomics than in bottom-up proteomics. In both cases, correct microorganism identification relies on the identification of unique versus degenerate biomarker ions. In this context, degenerate means either intact proteins (i.e., orthologs) or peptides found in two or more microorganisms.

3.3. Peptide Mass Maps

By analogy with intact protein mass searching for microorganism identification, the idea of utilizing the masses of proteolytic peptides generated in situ has been evaluated for identification of unfractionated viruses (101) and *Bacillus* spores (56). Databases were established against which experimentally generated suites of peptide masses were mapped. This approach provides successful identifications; however, the experimental step required for rapid residue-specific cleavage can be more productively linked with microsequencing by tandem MS, which allows more reliable protein and thus microorganism identification.

4. RAPID ANALYSIS OF NONGENOMIC CYCLIC LIPOPEPTIDES

Cyclic lipopeptides, e.g., polymixins, surfactins, fengycins, and kurstakins, are often abundant in bacteria and constitute a class of potential biomarker molecules that has been proposed to differentiate some microorganisms at the species and even at the subspecies levels (102–107). The structures of these secondary metabolites cannot be predicted from genome sequences, and it is necessary to employ library fingerprinting methods for identification. Both AP MALDI ion trap (23) and MALDI TOF/TOF (29) tandem spectra of these biomarkers are reproducible; therefore, it may be possible to incorporate tandem mass spectra for automated microorganism identification. For lipopeptides in which the ring structure is still intact, CID tandem MS results in fewer fragments, which reflect the structure of the fatty acid moiety (105).

5. AMPLIFYING PROTEIN SIGNALS FOR MASS SPECTRAL ANALYSIS

It has been recognized that MS, employing protein biomarkers for microorganism detection and identification, can never rival the sensitivity of PCR-based methods, which amplify the target DNA fragment many thousands of times. This extra sensitivity is especially important for the analysis of the complex mixtures of microorganisms typical in clinical infections. Recently, however, several MS-based functional assays have been developed and demonstrated for targeted bacteria, resulting in significant amplification of the signal.

5.1. Phage-Based Assay for Bacteria Identification

Targeted microorganisms have been identified by MALDI TOF MS through bacteriophage amplification (108). In this approach, samples containing pathogenic bacteria are infected with bacteria-specific bacteriophages (e.g., MS2 and MPSS-1 phages specific for *E. coli* and *Salmonella*, respectively). Proteins indicative of the progeny phages are then detected and utilized as secondary biomarkers for the target pathogen. For instance, *E. coli* mixed with both MS2 and MPSS-1 produces only an MS2 biomarker protein, as detected by MALDI TOF MS. Amplification of both phages in a mixture of the two bacteria leads to detection of biomarkers characteristic for both MS2 and MPSS-1.

5.2. Endopeptidase Assay for Anthrax

Anthrax lethal factor is a protein complex of a protective antigen and a zinc-dependent endoproteinase, which is known to cleave five protein kinases. The complex forms when the two proteins are secreted by the bacterium in an infected host. The protease activity of anthrax lethal factor has been exploited by Barr and colleagues for detection of anthrax in serum (109). Lethal factor is recovered from serum with monoclonal antibodies immobilized on magnetic protein G beads, and exposed to an optimized synthetic peptide substrate. The protease reiteratively hydrolyses peptide substrate molecules into two smaller peptides; this produces biomarkers amplified through time, which are detected by MALDI TOF mass spectrometry. Thus, three layers of selectivity contribute to the reliability of the analysis. The method can be carried out in four hours, with a detection limit of 0.05 ng/ml of the lethal factor complex. The cleavage reaction is shown in **Figure 5*a***, along with the masses of the protonated peptide products. **Figure 5*b*** shows the spectrum obtained from a control sample of peptide, processed in the absence of lethal factor. The ions detected represent the intact peptide with one and two charges. **Figure 5*c*** shows the spectrum obtained when 2 nmol of peptide is incubated with 1 ng lethal factor for two hours. The addition of known amounts of the peptide carrying stable isotope labels on the alanine residues has allowed quantitative analysis, and this assay is being used to study the progression of infection and disease in animals (109).

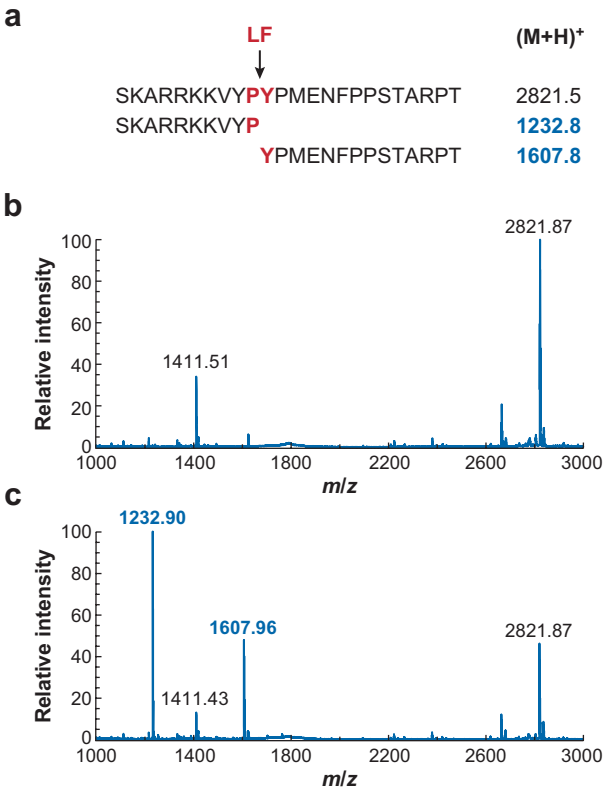

Figure 5

Anthrax lethal factor (LF) detection using an amplifying protease reaction. (*a*) The LF cleavage reaction of a target peptide with predicted masses of cleaved products denoted in the column. (*b*) Matrix-assisted laser desorption/ionization time-of-flight (MALDI TOF) spectrum of a peptide sample without addition of LF. (*c*) MALDI TOF spectrum of a peptide sample incubated with LF for 2 h. Reproduced with permission from Reference 109. Copyright 2007, American Chemical Society.

6. CONCLUSIONS

A number of modern MS instrumental systems—from MALDI TOF to tandem HPLC/ESI ion traps—have been applied for successful characterization of microorganisms at the species and, occasionally, the subspecies levels. Bioinformatics and proteomics approaches, initially developed for large-scale characterization of proteins, have been adapted to provide rapid and confident microorganism identification. Such approaches have a number of practical advantages; most notably, they do not require rigorous control of all culturing and experimental variables. Although the number of available prokaryotic genome sequences is still limited, the genomics community is moving aggressively to extend that inventory. In the meantime, it is clear that environmental, regulatory, and diagnostic analyses can be effectively carried out by MS using more limited or bounded sets of reference microbial genomes.

SUMMARY POINTS

1. MS determines with unprecedented accuracy an intrinsic molecular property: the masses of a set of biomarker molecules, which can uniquely characterize a microorganism.
2. Success has been demonstrated in the rapid characterization of microorganisms isolated/captured from various environments (e.g., air, water, culture medium, bodily fluids, and food) by mass spectrometry.
3. MS provides speed, sensitivity, and specificity for microorganism detection and identification. It is also broad band, and can be automated and multiplexed by, for instance, rapid screening of samples in 96- or 384-well plates. It is, however, expensive.
4. The applications of MS for microorganism characterization are significantly enhanced when it is combined with proteomic and bioinformatic strategies.

FUTURE ISSUES

1. Improved identification of individual microorganisms in mixtures (finding the "needle in the haystack") will be critical for future clinical applications.
2. Rapid characterization of bioengineered and mutated organisms is needed.
3. Contributions by MS will allow studies of microbial community metaproteomics to progress rapidly.
4. Rugged protein arrays (e.g., antibody or lectin), which can be read by MS, will be valuable for targeted applications.
5. Developments in microfluidics are expected to provide improved interfaces for sample preparation and transfer to ESI and MALDI.
6. Single microorganism trapping (e.g., in ion traps) and accurate mass determination will provide insights into the variability among individual cells and into the changes in total mass during, for instance, sporulation.

DISCLOSURE STATEMENT

A US patent (no. 7020559; listed as Reference 78 in this review) was issued in 2006. The patent rights holder is the University of Maryland. So far the patent has not been licensed.

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